Iranian Journal of Veterinary Science and Technology
Vol. 5, No. 1, 2013, 11-18

IJVST

Isolation of *Mycoplasma* spp. from broiler flocks with respiratory syndrome in Mashhad, Iran

Fatemeh Bibak[1], Gholam Ali Kalidari[2*], Jamshid Razmyar[2], Mehrnaz Rad[3],

[1]*Graduted from the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
[2]*Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
[3]*Department of Pathobiology, Faculty of Vetetinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*

Received: October 21, 2012 Accepted: February 12, 2013

Abstract

Mycoplasmosis is one of the most important diseases in the poultry industry. Its causative agent, mycoplasma has various species, which two of them, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are the most important species. Due to the enormous losses in the production farms of industrial poultry, achieving a rapid, accurate and definite diagnosis of mycoplasma is of great importance. An early and definite diagnosis can guarantee the farm management on keeping herd health. In many countries such as Iran, the disease and its complications have still remained as a serious problem. Given this issue, we decided to identify the mycoplasma infection from broiler poultry flocks through culture method. 150 carcasses of broiler chicken belonging to 50 broiler flocks were sampled in which the signs of air sacs involvement and secretions in the airways, trachea and bronchi were seen. Samples taken from trachea, palatine cleft, nasal passages and air sacs, were cultivated into PPLO liquid medium using membrane filters (0.45 micron). They were incubated at 37 °C and were examined for pH (color) changes for every 48 hours. During the first 24 hours after cultivation, every color change to yellow or dark visible with the bare eye was considered as bacterial contamination, therefore, the contaminated samples were removed from the incubator. The color change in the liquid media was compared with the uninoculated medium as negative control. If a color change was observed in the liquid media after 48h, subculture was done in the PPLO agar. The plates were incubated at 37 °C for 14 days. They were examined for mycoplasma colonies using a microscope with magnification of 10 in every other day. The results showed that out of 150 samples obtained from 50 broiler flocks, 16 (10.66%) were positive for mycoplasma, while in terms of contamination, 4 flocks (8%) were positive. The contamination of positive cultures was finally confirmed through PCR method with universal primer.

Keywords: Mycoplasmosis, PPLO medium, mycoplasma colonies, universal primer

* Corresponding author: Gholam Ali Kalidari
Email: kalidari@um.ac.ir
Fax: +98 51 38763852

Introduction

Mycoplasma infection has a worldwide importance due to the indication of significant economic losses in grandparents, breeders, broiler chicken and turkey rearing farms. Centers for Disease Control in Poultry have made many efforts to eradicate Mycoplasma infection in grandparent and breeder farms. However, in many countries such as Iran, Mycoplasmosis and economic losses resulting from it have still been remained as a serious problem (Stanley H. Celeven. 2008). Mycoplasmas are the simplest and smallest cells of prokaryote group and do not have cell walls. They are obligate parasites and live in the membranes of various vertebrates.

They are not much sustainable in medium and require a complex and dedicated medium containing 10 to 15 percent of pigs, horses or poultry serums that have been inactivated by heat for their growth. Mycoplasma colonies are small and have compressed and button-like cores and clear, vast, broad margin and are like scrambled eggs (Kleven, S.H. 1998). So far, 25 species of mycoplasma have been isolated from birds, but only four of those are known pathogenic. These include: *Mycoplasma gallisepticum* (MG) and *Mycoplasma Synoviae*, which cause disease in poultry and turkey. *Mycoplasma meleagridis* and *Mycoplasma iowae* are pathogenic for turkeys (Bradbury J m. *1998*). Since there is the possibility for occurrence of simultaneous infection of various mycoplasma species in a host, the set of clinical signs of cattle is observable. Achieving rapid, accurate and decisive diagnosis is very important for health authorities and veterinarians of breeders and grandparent farms due to its economic importance. Since, early and definitive diagnosis can help the hygiene management of farms in maintaining the public health of flock, prevention from disease progression and choosing the good regulatory policy is significant (Razin S., Yogev D, Naot Y. 1998).

If the infection in the breeder flocks has been detected, house birds or involved houses were removed and greater care and protection (bio- security) measures are applied and controlled in non-polluted farms. Also due to the significant difference of price between pullets and infected- and non-infected chickens, determining the *Mycoplasma gallisepticum* status in these flocks has been as a usual and common plan (Kleven, S.H. 1998). Apart from the serological testing of broiler chickens in the ages between one to four days, there is not another routine monitoring to determine the status of *Mycoplasma gallisepticum* infection in the broiler flocks in Iran; especially it is more likely to contaminate the broiler chickens in the ages between 4 to 8 weeks (Kleven, S.H. 1998). Due to mutual interference phenomenon with other mycoplasma species and also the presence of variable non-specific factors in serum, serologic tests do not have sufficient sensitivity and specificity for the diagnosis of mycoplasma strains and sometimes cause false positive responses, whereas mycoplasma needs long time for optimal growth. The isolation and cultivation of mycoplasma have been used as a definitive method of final confirmation of poultry mycoplasmosis (Bradbury J m. 1998 & Hanif A, Najeeb M.I. 2007).

Materials and methods

Sampling

150 carcasses of broiler chicken belonging to 50 broiler flocks were sampled in which the air sacs involvement were seen and secretions in the airways, trachea and bronchi, and the samples of trachea, palatine cleft, nasal passages and air sacs were received (Khiari A. *et al*., 2011)

The samples were taken from the cases, which referred to the poultry division of Ferdowsi University of Mashhad, and also from private clinics. Carcasses of broiler chickens in which there were involvement of air sacs randomly were chosen and about 4 to 6 samples were in average obtained from every flock (Ghaleh Golab Behbahan N,

2005).

Data forpoultry flocks were fullyrecorded. Information related to each poultries including sampling date, poultry capacity, and the type of breeding, the number of deaths, the age of involvementand the number of samples was recorded.For sampling, at first,the external part of carcasses was cleaned and disinfected with alcohol. Then required parts were cut with scissors to access air sacs, trachea and palatine cleft.Then, trachea cut with sterile scissors and a sterile swab was entered into the trachea secretions and sampled, then, swab was taken in tubes containing physiologic serum. Another swab was taken into nasal duct and sampled from nasal secretions and swap was put in the tube. Again, anotherswab was taken from the inside of palatine cleft and the latter taken from air sacs and finally, the samples in ice were transported to the laboratory (Kleven, S.H. ,1998 & Khiari A, 2011).

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Culture procedure

Tube containing sample swabs were put on a shaker.Then,two swabs with 0.45 microns membranes filter were put in PPLO liquid medium with NAD and Cysteine and two other swabs were inoculated in PPLO liquid medium without NAD and Cysteine (Stanley, H and Celeven, 2008). All liquid media were incubated at 37 °C(4, 1).The media were daily examined.During the first 24 hours, color change or dark was considered bacterial contamination and were removed from the incubator in the next days, color changed from red to yellow, the culturewas immediately transferred to the brothmedium. The samples without any color change up to 10 days were also transferred to the broth medium. Broth medium were remained in the incubator and in the seventeenth day, the samples with color change were transferred to the agar medium by using membrane filter (Kleven, S.H. 1998). The original samples were kept in the incubator for a month and if there was not any evidence of growth, they were considered asnegative (5, 4). Agar media were incubated at 37 °C for7 to 10 days. They were examined for colonies in microscope.(5, 4).To check for any contamination with bacteria other than mycoplasma and fungi in liquid media, aliquots of color changed liquid media were inoculated on the blood agar (Hanif A and Najeeb M.I.2007&Yilmaz F 2011).

DNA extraction and PCR

DNA was extracted from colonies by boiling (Pourbakhsh S.A 2010 &Wong-Lee JG 1993).

Bacterial suspensions were prepared in normal saline and were centrifuged at7000g (5 minutes). After 2 or 3 times washing steps with normal saline, the supernatant was removed and discarded. Then, 200ml sterile distilled water or TE was added to the contents deposited in the microtube and was boiled for about 5 minutes at 100 C and immediately placed in a freezer. Freezing and boiling process were repeated 3 times. The contents of the microtube were centrifugedat7000g and the supernatant containing bacteria lDNA was transferred to another microtube. Universal primers described by Wong-Lee andLovett were chosen in this study (Wong-Lee JG, Lovett M , 1997). Forward primer 5-GGCGAATGGGTGAGTAACACG-3 and reverse primer 5-ATAGGTCGCAAGCGTTATCCG-3 with 464 bp PCR product size because these universal primers allowed detection of most mycoplasma species. The amplification mixtures consisted of a 25µL reaction mix of 2.5µL PCR buffer (10X), 0.5µLof dNTPsmix(10mM), 1µL of MgCl2(50mM), 0.2µL DNA Polymerase(5U/µL), 10pmol of each primer, 5µL DNA sample, and 13.3µL of DW. Cycling Amplification conditions involved a 5 min denaturing step at 94°C, followed by 35 cycles of denaturation at 94°C for30 s, annealing at 60°C for 30 s, and a primer extension at 72°C for 30 s. After the last cycle the product was incubated at 72°C for 5 min. The PCR products were subjected

to electrophoresis in 1.5 % (w/v) agar gels in 1×TAE buffer (40 mMTris- acetate, 1mM EDTA). The voltage for electrophoresis is set at 60 Volts for 70 min. The bands were stained by emersionin 0.5µg/ml ethidiumbromide for 30 min, and the gels were viewed and recorded by geldocumentation apparatus (Wong-Lee JG 1993).

Results

Out of 150 samples obtained from 50 broiler flocks, 16 (10.66%) mycoplasma isolates were obtained from the samples and 4 (8%) flocks were positive for mycoplasma. All samples related to these four flocks, which were reported as positive, werefinally confirmed by PCR method with universal primer (Figure 1 & 2 and Tables 1).

Table 1.Results of the isolation of Mycoplasma spp.

Flock number	No. of positives in Broth and Solid PPLO media	Genus specific PCR
40	6	Done
38	5	Done
29	2	Done
26	3	Done
Total isolates	16	

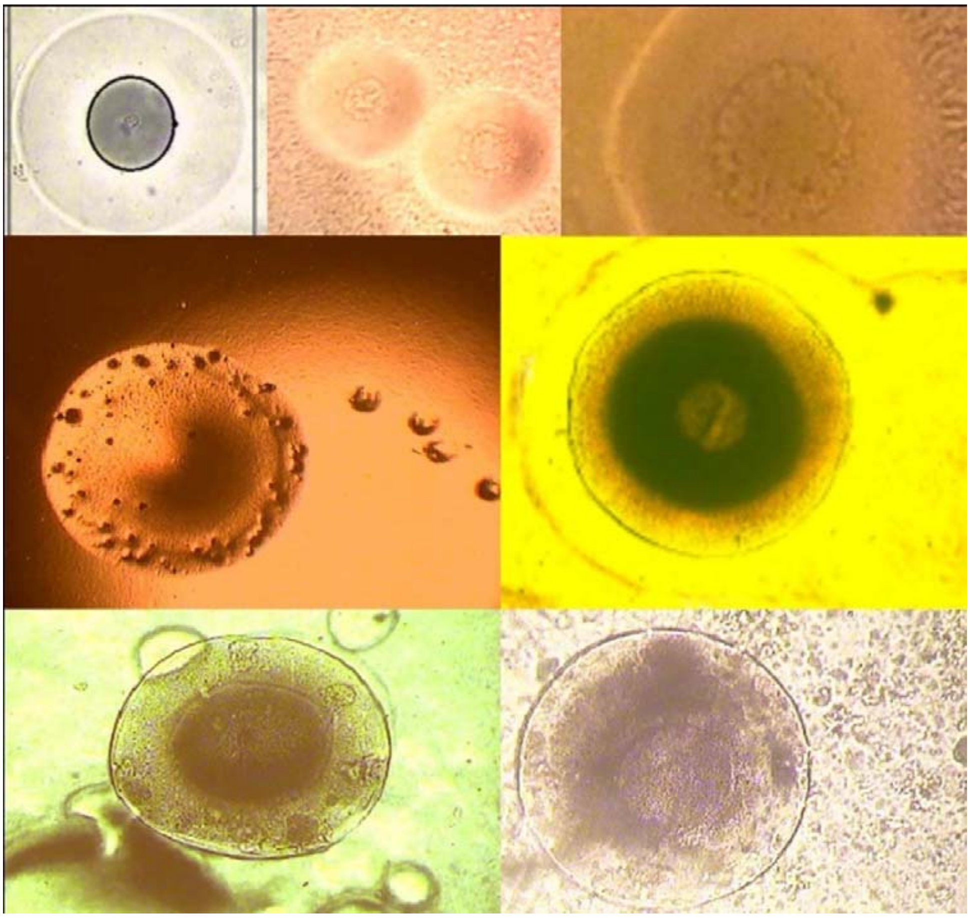


Figure 1. Mycoplasma colonies in this study. X * 100

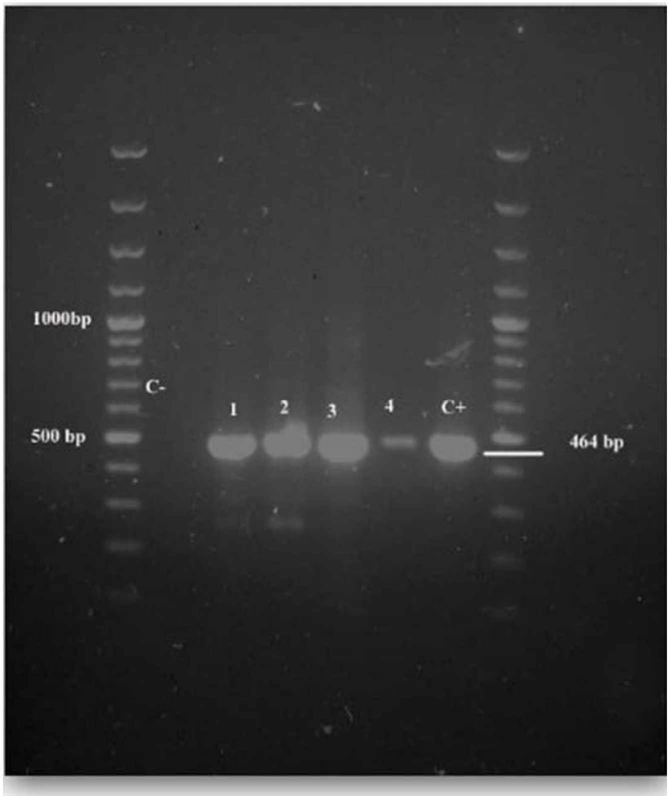


Figure 2. Photograph of 1% agarose electrophoresis gel showing the PCR amplification reaction products using various DNA samples. Lane 1: 100 bp ladder, lane 2: C-negative control, lane 3 to 5: positive samples, lane 6: C+ (positive control from the vaccinal strain of Mycoplasma synoviae).

Discussion

The lack of mycoplasma growth in a rich growth medium is not the result of the lack of a specific nutrient but it results from the presence of toxic combination or combinations for mycoplasma. It is believed that non-cultivable mycoplasma strains are not certain strictstrains but they are more sensitive to available inhibitors in the medium particularly, in compounds such as peptone and yeast extract. Therefore, two terms, non-cultivable and strict are relative terms which are only interpretable about a particular cultivation system in which there are motivators and possible growth inhibitors (Kleven, S.H. 1998). One of the important issues in isolating avian pathogenic mycoplasma is includes attention to the presence of saprophytic mycoplasmas especially *Mycoplasma gallinarum and Mycoplasma gallinaceum*. The growth of these mycoplasmas are much faster than pathogenic mycoplasmas (Khiari A *et al*., 2011). Due to the lack of regular serological monitoring in laying and broiler flocks,the majority of these flocks are chronically suffering disease and despite positive serologic tests, mycoplasma isolation is hard.

In one study, out of 463 obtained samples, only (4.8%) isolation was possible (Firits *et al*., 1991). In another study revealed that in 10 broiler, laying, broilerbreeder and laying breeder seropositive flocks, from a total of 404 samples, *mycoplasma* from 131 (32.4%) samples were isolated (Hosseini, H and Bozorgmehrifard M.H , 2007).

Isolation rate of (11.1%) MG in three flocksand (44.4%) in 12 flocks out of 27 serologically positive broiler flocks was reported (Yilmaz, F *et al*., 2011). In 2010, isolated 7 (30.4%) *Mycoplasma gallisepticum* out of 23 broiler breeder flocks (Purbakhsh *et al*., 2010). In 2011, *Mycoplasma meleagridis* isolation from 10 flocks (33.3%) out of 30 serologically positive broiler flocks was

reported (Khiari, A *et al*., 2011) that means using species-specific tools such as PCR is crucial to determine exact cuase of seropsitive reactions in rapid slide tests.

The higher percentage of mycoplasma in cultivation in other studies might be due to this fact that the isolation in these works had been done from the flocks in which, their mycoplasma contamination had been approved early in terms of serology, but in our study , the flocks were randomly sampled regardless to selogical results . Out of 50 studied flocks, 4 (8%) flockswere positive and mycoplasma spp were isolated. To enhance the chances of isolation, primary cultivation and serological tests must be done and PCR from samples are recommended for confirmation.

Acknowledgements

The authors whould like to appreciate Mr. Ali Kargar for technical helps. The expenses of this study were also founded through research No.17410 research deputy of Ferdowsi University of Mashhad which is hereby appreciated.

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Iranian Journal of Veterinary Science and Technology
Vol. 5, No. 1, 2013, 11-18

IJVST

جداسازی مایکوپلاسما از گله های جوجه گوشتی در مشهد،ایران

فاطمه بی باک[1]، غلامعلی کلیدری[2*]، جمشید رزمیار[2]، مهرناز راد[3]

[1]دانش آموخته دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
[2]گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
[3]گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران

دریافت مقاله : ۱۳۹۱/۰۷/۳۰ پذیرش نهایی: ۱۳۹۱/۱۱/۲۴

چکیده

بیماری مایکوپلاسموز طیور یکی از بیماری های مهم در صنعت طیور است.عامل این بیماری مایکوپلاسما بوده که دارای گونه های مختلف است.دوگونه مایکوپلاسماگالیسپتیکوم و مایکوپلاسماسینویه از بین دیگرگونه‌ها درایجاد ضرر وزیان در پرورش طیور ٠صنعتی مهمتر می باشد. در این بررسی جهت جداسازی و شناسایی مایکوپلاسما از ۱۵۰ لاشه ی مرغ گوشتی متعلق به۵۰ گله ی گوشتی که در آنها درگیری کیسه های هوایی و ترشحات در مجاری هوایی ، نای و برونش ها دیده می شد نمونه گیری انجام شد. نمونه ها از نای، شکاف کامی، مجرای بینی و کیسه های هوایی اخذ شده و پس از جمع آوری با فیلتر ۰/۴۵ میکرون به داخل محیط کشت مایع PPLO کشت منتقل و داخل انکوباتور ۳۷ درجه قرار داده شد. محیط ها هر ۴۸ ساعت جهت مشاهده تغییر رنگ محیط از قرمز به سمت زرد مورد بررسی قرار می گرفت. طی ۲۴ ساعت اول بعد از کشت، هر تغییر رنگی به زرد یا کدورت قابل مشاهده با چشم غیر مسلح ناشی از آلودگی باکتریایی قلمداد شده و نمونه‌های آلوده از انکوباتور حذف می شدند. تغییر رنگ ایجاد شده در محیط های کشت مایع، با عدم تغییر رنگ نمونه کنترل که به عنوان کنترل منفی در نظر گرفته شده بود مقایسه می شد. در صورت مشاهده تغییر رنگ در محیط های کشت مایع، تجدید کشت در محیط جامد PPLO صورت می گرفت. از روز چهارم به بعد بطور یکروز درمیان محیط جامد جهت مشاهده پرگنه های مایکوپلاسما در زیر میکروسکوپ با درشت نمایی ۱۰ بررسی می شد. نتایج حاصله مشخص کرد که از کشت تعداد ۱۵۰ نمونه اخذ شده از ۵۰ گله ی گوشتی، تعداد ۱۶ جدایه مایکوپلاسما (۱۰/۶۶٪) از نمونه ها حاصل شد و تعداد ۴ گله(۸٪) از ۵۰ گله ی مورد مطالعه از نظر آلودگی به مایکوپلاسما مثبت بودند. با استفاده از روش PCR با پرایمر یونیورسال نتایج مثبت به دست آمده تایید نهایی شد.

واژگان کلیدی: بیماری مایکوپلاسموز، محیط کشت PPLO، کلنی های مایکوپلاسما، پرایمر یونیورسال